Production of Long-chain Levan by a sacC Insertional Mutant from Bacillus subtilis 327UH

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A hyper extracellular protein producer, Bacillus subtilis 327UH, produced large amounts of levan in a medium containing 20% sucrose, and the yield of levan after 10 hours was more than 60%, when based on the fructose amount of sucrose. After transformation of 327UH with a levanase-deficient 168SC (sacC::Cmr) chromosomal DNA, a Cm:r transformant 327UHSC (sacC::Cmr degSU(Hy)) produced 3 times longer levan than that of the wild type.

Key words: levan; levanase; sacC; degU-hyper strain; Bacillus subtilis

Levan and its oligosaccharides are widely distributed and produced by various microorganisms1–5) and plants. They are storage compounds in plants,7) a soil aggregation compound in the wheat rhizosphere,8) and an antitumor polysaccharide.9) B. subtilis also produces levan the structure of which has been reported by Rapoport and Dedonder.10) Levan-sucrases, levan-producing enzymes, and their genes are reported in various microorganisms (Fig. 1).1,11,12) B. subtilis produces a levan-sucrase (SacB)1,13) and the degSU-hyper mutations which have been used for hyperproduction of α-amylase and proteases also stimulate levan-sucrase expression at the transcription level.14,15) Levanase, an β-2,6-fructofuranoside linkage-hydrolyzing enzyme, and its gene (sacC) have also been reported in B. subtilis,16) but there is almost no approach to produce larger amounts of levan with high molecular mass not only in B. subtilis, but also in other microorganisms. Since levan is a natural and safe polysaccharide, new uses for biofiber and biopolymer materials will be interesting. Therefore we attempted to produce levan in large quantities and to manipulate the degree of polymerization.

B. subtilis 168 was a parent strain and 327UH was a degU32(Hy) mutant producing large amounts of α-amylase and proteases.14,15) Escherichia coli JM109 (Takara) was used as a cloning host. pUC18 (Takara) and pMWEC17) were E. coli vectors to disrupt the sacC gene. E. coli and B. subtilis were cultured in LB agar medium, if necessary supplemented with 5 μg/ml chloramphenicol and/or 1% (w/v) casein. For preculture of B. subtilis 327UH and 327UHSC (described below), C medium (70 mM K2HPO4, 30 mM KH2PO4, pH 7.0, 25 mM (NH4)2SO4, 0.5 mM MgSO4, 0.01 mM MnSO4, 22 mg/l ammonium iron

Note

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(III) citrate, and 0.2% yeast extract (Wako) containing 0.4% glucose was used. For main culture, C medium containing 2% yeast extract and 20% sucrose was used.

To construct the sacC::Cm' disruptant of B. subtilis 327UH, the sacC gene fragment was amplified from the chromosomal DNA of B. subtilis 168 by PCR using ExTaq polymerase (Takara). The primers used for the PCR were d-CCGCGAATTCC AAGCCCTTTGCTTGATCGGTCTG and d-GGCGAAGCTTGCGTCAGCAAAGATTTGGTTACAATGG, in which the sacC sequences are italicized and restriction endonuclease sites are underlined. The sacC gene fragment was ligated to the EcoRI and the HindIII sites of pUC18, and the resulting plasmid, pUCS1, was obtained after transformation of E. coli JM109 cells. The Cm' gene, which was inserted at the SphI site of the sacC gene fragment, was amplified from pMWEC1(15) by PCR using primers containing SphI site. The primers were d-GGCTGCAATGGCCACACCTTAC and d-CCGAGCAGCTGCAACTAACGGGGCAGGTTTGCACGGTTACAATGG, in which the Cmr sequences are italicized and SphI sites are underlined. The amplified Cm' gene was cloned at the SphI site on the sacC gene fragment of pUCS1. The desired plasmid pUCSC1 was obtained from transformed E. coli JM109 cells.

Since the efficiency of the transformation of B. subtilis 327UH with a linearized plasmid by double crossing-over integration was very low, we attempted to get a transformant of B. subtilis 168. The plasmid pUCSC1 was linearized by Scal digestion and then competent B. subtilis 168 cells were transformed by double cross-over integration using the Scal-digested DNA. The sacC::Cm' disruptant, B. subtilis 168SC (sacC::Cm'), was selected on a LB agar plate containing chloramphenicol. After confirmation of the gene structure of sacC::Cm' in B. subtilis 168SC, B. subtilis 327UH (degU32(Hy)) was transformed by B. subtilis 168SC chromosomal DNA. Since a strain containing the degU32(Hy) mutation hyperproduces various extracellular enzymes including proteases and levansucrase, (16) a chromlamphenicol-resistant transformant, B. subtilis 327UHSC (degU32(Hy) sacC::Cm'), was selected on an LB agar plate containing chloramphenicol and casein. B. subtilis 327UHSC formed a large halo (indicating casein-hydrolyzing activity) that is equal to that of B. subtilis 327UH.

B. subtilis 327UH and 327UHSC was first cultured in C medium (25 ml) containing 0.4% glucose at 37°C for 8 h. The main culture (400 ml) in a 3-liter Sakaguchi flask was started by adding 1% of the first culture to C medium containing 2% yeast extract and 20% sucrose, and incubated at 37°C with air-bubbling (0.8 vvm). To each culture supernatant (10 ml) obtained by centrifugation (7,500 rpm, 5 min, room temperature) was added 95% ethanol (20 ml). The precipitated levan was collected by centrifugation (9,000 rpm, 5 min). The pellet was re-dissolved in deionized water (10 ml) and levan was re-precipitated with 95% ethanol (20 ml). This step was repeated twice. The collected levan was dissolved in water (2 ml) and then lyophilized.

Figure 2 shows the courses of cell growth and yield of levan. B. subtilis 327UH produces levan from sucrose at the beginning of the transitional phase of growth, and reached a maximum in the early stationary phase. The yield of levan was more than 60% in C medium containing 20% sucrose. No difference between B. subtilis 327UH and 327UHSC was detected in their tendencies of growth and yields of levan. It was obvious that the growth and the levan productivity of B. subtilis 327UHSC were not affected by the disruption of the sacC gene. To analyze the polymerization degree of levan, we prepared levan from a 10-h culture of each strain (B. subtilis 327UH and 327UHSC). The 1H-NMR spectra of levan and sucrose were obtained with a Bruker DPX-400 spectrometer (data not shown). The chemical shifts of the H-1 resonances of the α-D-glucopyranosyl moieties were 5.402 for sucrose and 5.403 and 5.369 for levan. By considering that the H-1 resonance of 1-kestose is larger than that of 6-kestose, (19,20) it was speculated that the chemical shift of levan containing 6-kestose as a terminal residue is 5.369 ppm and the chemical shift of levan containing 1-kestose as a terminal residue is 5.403. The ratio of both terminal kestose residues was nearly equal. By the way, the core of this study is that two types of levan were produced by levanase (Fig. 1). Type I levan was made of fructose only and had a reducing terminus. Type II was a fructose polymer with a glucose residue. In the type II levan, the glucose residue binds to the terminal fructose by α-glycoside bond. Therefore, type II
Effects of Levanase on Levan Production by B. subtilis

Table 1. Ratios of Constituent Monosaccharides and Polymerization Degree of the Collected Levans

<table>
<thead>
<tr>
<th>Constituent Monosaccharides¹,b</th>
<th>B. subtilis 327UH</th>
<th>B. subtilis 327UHSC (sacC::Cmr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fructose (with reducing power)</td>
<td>10.2</td>
<td>36.1</td>
</tr>
<tr>
<td>Monosaccharides (glucose + fructose)</td>
<td>120</td>
<td>1230</td>
</tr>
<tr>
<td><strong>Average Polymerization Degree</strong>e</td>
<td>10.7</td>
<td>33.2</td>
</tr>
</tbody>
</table>

a: The collected levan (0.1 g) was dissolved in 1 N HCl (400 μl) and incubated at 70°C for 3 h. Using TLC analysis, it was confirmed that the levan was completely hydrolyzed to monosaccharides. The constituent monosaccharides were analyzed by a high-performance liquid chromatograph equipped with a reducing sugar analysis system LC-10Advp (Shimadzu, Kyoto). Conditions of TLC and HPLC are as follows. Whatman K5F TLC plates were developed in n-butanol:2-propanol:acetic acid (7:5:4:2, v/v/v/v) and spots were made visible by spraying a p-anisaldehyde solution (p-anisaldehyde:conc. H2SO4:ethanol; 1:1:18, v/v/v) followed by heating at 90°C for 5–10 min. For HPLC analysis, monosaccharides were chromatographed on a Shim-pack Isa-07s column (4 mm × 250 mm) with a pH gradient from pH 8 to pH 9 in 0.1 M borate acid at a flow rate of 0.6 ml/min at 65°C. Eluted monosaccharides were post-labelled by heating in 1% arginine and 3% boric acid at 150°C and absorbance at 430 nm was recorded under 320 nm excitation.

b: The amount of the constituent monosaccharides is represented by the relative amount of glucose.
c: The amount of the terminal fructose residue with reducing power of the levan was measured by the Park-Johnson method.²²

d: The gross total of the constituent monosaccharides.
e: The average polymerization degrees of the collected levans were calculated by dividing the gross total of the constituent monosaccharides with the sum of the terminal glucose and the terminal fructose with reducing power.

levan does not have reducing power. The number of levan molecules is equal to the sum of the number of fructose residues with reducing power and glucose residues. Consequently, the mean of the polymerization degree of isolated levan was obtained by dividing the number of fructose and glucose residues by the sum of the number of glucose residues and fructose residues with reducing power. Table 1 shows the mean of the polymerization degree of levan produced by B. subtilis 327UH and 327UHSC. The polymerization degree of levan obtained from B. subtilis 327UHSC was increased approximately three times, compared with that of B. subtilis 327UH. Therefore it is very likely that the increase of the polymerization degree of levan was attributable to the deletion of the SacC protein. However, on the basis of the polymerization degree the terminal fructose residue with reducing power, it is suggested that B. subtilis produces another levanase. The yveB gene is a candidate, because yveB shows 36% amino acid sequence similarity with sacC over 494 amino acid residues (BSORF database). The other significant similarity is with the sacA gene, but it is reported to be a sucrose-6-phosphate hydrolase.²² Therefore, an additional disruption of yveB will become an interesting approach to make levan much longer.

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References

12) Yanase, H., Fujimoto, J., Maeda, M., Okamoto, K.,


